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(54) IMPROVED METHODS OF USING CHEMILUMINESCENT 1,2-DIOXETANES.

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Description**FIELD OF THE INVENTION**

5 This invention relates to improved methods of using chemiluminescent compounds, and especially enzymatically cleavable chemiluminescent 1,2-dioxetane compounds. More particularly, this invention relates to the generation and detection of electromagnetic energy released by the decomposition of enzymatically cleavable and chemically cleavable chemiluminescent 1,2-dioxetane compounds used to determine the presence, concentration or structure of substances in a sample, especially an aqueous
 10 sample, particularly when such chemiluminescent compounds are used to detect the presence or determine the concentration of chemical or biological substances by art-recognized immunoassay techniques, chemical assays or nucleic acid probe assays, or when they are used as direct chemical/physical probes for studying the molecular structures or microstructures of various macromolecules: synthetic polymers, proteins, nucleic acids and the like.

BACKGROUND OF THE INVENTION

The decomposition of chemiluminescent chemical compounds to release electromagnetic energy, and especially optically detectable energy -- usually luminescence in the form of visible light -- is well known
 20 and understood. The incorporation of such light emitting reactants in art-recognized immunoassays, chemical assays, nucleic acid probe assays and chemical/physical probe techniques as the means by which the analyte, a substance whose presence, amount or structure is being determined, is actually identified or quantified has assumed increasing importance in recent years, particularly with the advent of enzymatically-cleavable 1,2-dioxetanes.

SUMMARY OF THE INVENTION

The present invention is based upon the discovery that by using two or more enzymatically cleavable (decomposable) chemiluminescent 1,2-dioxetane compounds, such compounds being configured, by means
 30 of the inclusion of a different light-emitting fluorophore moiety in each molecule, to emit light of a different wavelength from the other(s) upon decomposition [e.g., one such compound can contain a fluorophoric coumarin (benzopyranyl) residue unsubstituted except for a labile ring substituent such as a phosphate ester or acetate ester group, e.g., dispiro(adamantane-2)-3'-(1',2'-dioxetane)-4',2''-(7''-phosphoryloxy-3''-chromene) sodium salt; the other(s) a labile ring substituent-containing fluorophoric trifluoromethyl- or
 35 benzothiazolylbenzopyranyl residue], and each such compound being structured so as to be cleavable by a different enzymatic cleaving means [e.g., one such compound can contain, as mentioned above, a phosphate ester group cleavable by a phosphatase or an acetate ester group cleavable by a carboxylesterase, the other(s) can contain an α -D- or β -D-glucoside group cleavable by a glucose oxidase or a β -D-galactoside group cleavable by a β -galactosidase], light of different wavelengths can be induced
 40 simultaneously or sequentially by the decomposition of these differently configured and differently decomposable chemiluminescent compounds. Hence, multi-channel assays can be designed in which different enzymes attached to or associated with two or more different analytes will, by cleaving different enzyme cleavable dioxetane substituents, induce the emission of light of a different wavelength for each analyte being assayed.

45 Further, the emission of light of different wavelengths by a multiplicity of decomposable chemiluminescent compounds, e.g., in multi-channel assays, can also be accomplished by using one or more enzymatically cleavable chemiluminescent 1,2-dioxetane compounds and one or more chemically or electrochemically cleavable chemiluminescent compounds, which for example contain, instead of an enzyme cleavable group, a chemically cleavable group such as a hydroxyl group, an alkanoyl or aroyl ester group
 50 such as an acetoxyl group, or an alkyl or aryl silyloxy group such as a t-butyltrimethylsilyloxy or t-butylphenylsilyloxy group, together with one or more enzymes and one or more chemical cleaving means, each attached to a different substance, e.g., an analyte, to once again induce the emission of light of a different wavelength from each such decomposable chemiluminescent compound, e.g., for each analyte being assayed in a multichannel assay.

55 It is, therefore, an object of this invention to provide improved methods of using chemiluminescent 1,2-dioxetane compounds, and especially enzymatically cleavable chemiluminescent 1,2-dioxetane compounds.

A further object of this invention is to provide improved methods of inducing the simultaneous generation of light of different wavelengths by decomposing differently configured and differently decom-

possible chemiluminescent compounds, including chemiluminescent 1,2-dioxetane compounds and, in particular, enzymatically cleavable chemiluminescent 1,2-dioxetane compounds.

A still further object of this invention is to provide multi-channel assays carried out in the presence of at least two differently configured and differently decomposable chemiluminescent compounds, including chemiluminescent 1,2-dioxetane compounds and, in particular, enzymatically cleavable chemiluminescent 1,2-dioxetane compounds, as substrates, each of which compounds emits light of a different wavelength from the other(s) and each of which has a labile substituent cleavable by a different means from the other(s), to detect the presence or determine the concentration, by art-recognized immunoassay, chemical assay and nucleic acid probe assay techniques, of chemical or biological substances, and to elucidate the molecular structures or microstructures of various macromolecules: synthetic polymers, proteins, nucleic acids and the like, by art-recognized direct chemical/physical probe techniques.

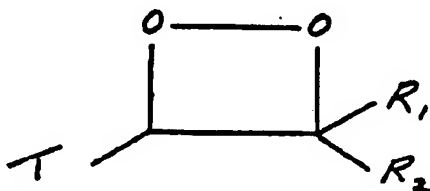
These and other objects, as well as the nature, scope and utilization of this invention, will become readily apparent to those skilled in the art from the following description and the appended claims.

15 DETAILED DESCRIPTION OF THE INVENTION

Suitable enzymatically cleavable chemiluminescent 1,2-dioxetane compounds can be represented by the general formula:

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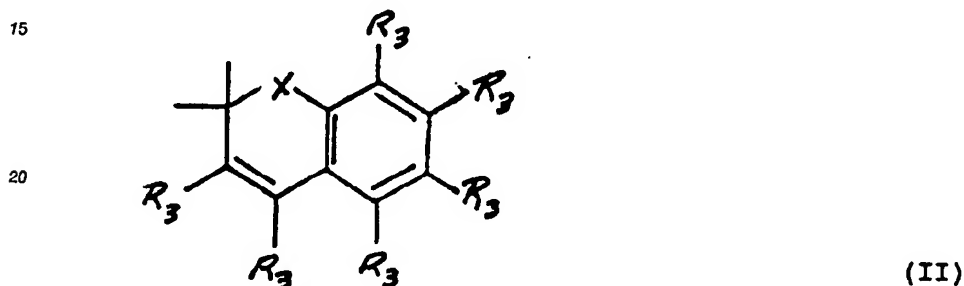
In this formula the symbol R_1 represents hydrogen, or a bond when R_2 represents a substituent bound to the dioxetane ring through a spiro linkage, or an organic substituent that does not interfere with the production of light and that satisfies the valence of the dioxetane ring carbon atom to which it is attached to result in a tetravalent dioxetane ring carbon atom; such as an alkyl, aryl, aralkyl, alkaryl, heteroalkyl, heteroaryl, cycloalkyl or cycloheteroalkyl group, e.g., a straight or branched chain alkyl group having from 1 to 7 carbon atoms, inclusive; a straight or branched chain hydroxyalkyl group having from 1 to 7 carbon atoms, inclusive, or an -OR group in which R is a C_1 - C_{20} unbranched or branched, unsubstituted or substituted, saturated or unsaturated alkyl, cycloalkyl, cycloalkenyl, aryl, aralkyl or aralkenyl group, any of which may additionally be fused to R_2 such that the emitting fragment contains a lactone ring, a fused ring cycloalkyl, cycloalkenyl, aryl, aralkyl or aralkenyl group, or an N, O or S heteroatom-containing group; or a light-emitting fluorophore-forming fluorescent chromophore group bonded to the dioxetane ring through a single bond or a spiro linkage, i.e., a group capable of absorbing energy to form an excited energy state from which it emits optically detectable energy to return to its original energy state, to which an enzyme-cleavable group is bonded by a bond cleavable by an enzyme to yield an electron-rich moiety bonded to the dioxetane ring, e.g., a bond which, when cleaved, yields an oxygen anion, a sulfur anion or a nitrogen anion, and particularly an amido anion such as a sulfonamido anion. Preferably, R_1 is a methoxy group.

The symbol R_2 represents hydrogen, or a bond when R_1 represents a substituent bound to the dioxetane ring through a spiro linkage, or a light-emitting fluorophore-forming fluorescent chromophore group, bonded to the dioxetane ring through a single bond or a spiro linkage, to which an enzyme-cleavable group is bonded by a bond cleavable by an enzyme to yield an electron-rich moiety bonded to the dioxetane ring, e.g., one of the aforementioned bonds which, when cleaved, yields an oxygen, sulfur or nitrogen anion.

The light-emitting fluorophore-forming fluorescent chromophore groups which can be symbolized by R_1 or R_2 can be the residues of the auxiliary fluorophores listed below, unsubstituted or substituted with one or more non-labile substituents such as a branched or straight chain alkyl group having 1 to 20 carbon atoms, inclusive, e.g., methyl, n-butyl or decyl; a branched or straight chain heteroalkyl group having 1 to 7 carbon atoms, inclusive, e.g., methoxy, hydroxyethyl or hydroxypropyl; an aryl group having 1 or 2 rings, e.g., phenyl; a heteroaryl group having 1 or 2 rings, e.g., pyrrolyl or pyrazolyl; a cycloalkyl group having 3 to 7 carbon atoms, inclusive, in the ring, e.g., cyclohexyl; a heterocycloalkyl group having 3 to 6 carbon atoms, inclusive, in the ring, e.g., dioxane; an aralkyl group having 1 or 2 rings, e.g., benzyl; an alkaryl group

having 1 or 2 rings, e.g., tolyl; an electron-withdrawing group, such as a perfluoroalkyl group having between 1 and 7 carbon atoms, inclusive, e.g., trifluoromethyl; a halogen; CO₂H, ZCO₂H, SO₃H, NO₂, ZNO₂, C≡N, or ZC≡N, where Z is a branched or straight chain alkyl group having 1 to 7 carbon atoms, inclusive, e.g., methyl, or an aryl group having 1 or 2 rings, e.g., phenyl; an electron-donating group, e.g., a
 5 branched or straight chain C₁-C₇ alkoxy group, e.g., methoxy or ethoxy; an aralkoxy group having 1 or 2 rings, e.g., phenoxy; a branched or straight chain C₁-C₇ alkoxy group, e.g., methoxy or ethoxy; an aralkoxy group having 1 or 2 rings, e.g., phenoxy; a branched or straight chain C₁-C₇ hydroxyalkyl group, e.g., hydroxymethyl or hydroxyethyl; a hydroxyaryl group having 1 or 2 rings, e.g., hydroxyphenyl; a branched or
 10 straight chain C₁-C₇ alkyl ester group, e.g., acetate; an aryl ester group having 1 or 2 rings, e.g., benzoate; or a heteroaryl group having 1 or 2 rings, e.g., benzoxazole, benzthiazole, benzimidazole or benztriazole.

The symbols R₁ and R₂, taken together, can be a fused fluorescent chromophore group bonded to the dioxetane ring through a spiro linkage, e.g., one having the general formula:



25 In this formula X is



- O -, - S - or -NR₆ where each of R₄, R₅ and R₆, independently, is hydrogen, a branched or straight chain alkyl group having 1 to 20 carbon atoms, inclusive, e.g., methyl, n-butyl or decyl, a branched or straight
 35 chain heteroalkyl group having 1 to 7 carbon atoms, inclusive, e.g., methoxy, hydroxyethyl or hydroxypropyl; an aryl group having 1 or 2 rings, e.g., phenyl; a heteroaryl group having 1 or 2 rings, e.g., pyrrolyl or pyrazolyl; a cycloalkyl group having 3 to 7 carbon atoms, inclusive, in the ring, e.g., cyclohexyl; a heterocycloalkyl group having 3 to 6 carbon atoms, inclusive, in the ring, e.g., dioxane; an aralkyl group having 1 or 2 rings, e.g., benzyl; an alkaryl group having 1 or 2 rings, e.g., tolyl; or an enzyme-cleavable
 40 group as defined above; and each R₆, independently, can be hydrogen; an electron-withdrawing group, such as a perfluoroalkyl group having between 1 and 7 carbon atoms, inclusive, e.g., trifluoromethyl; a halogen; CO₂H, ZCO₂H, SO₃H, NO₂, ZNO₂, C≡N, or ZC≡N, where Z is a branched or straight chain alkyl group having 1 to 7 carbon atoms, inclusive, e.g., methyl, or an aryl group having 1 or 2 rings, e.g., phenyl;
 45 an electron-donating group, e.g., a branched or straight chain C₁-C₇ alkoxy group, e.g., methoxy or ethoxy; an aralkoxy group having 1 or 2 rings, e.g., phenoxy; a branched or straight chain C₁-C₇ hydroxyalkyl group, e.g., hydroxymethyl or hydroxyethyl; a hydroxyaryl group having 1 or 2 rings, e.g., hydroxyphenyl; a branched or straight chain C₁-C₇ alkyl ester group, e.g., acetate; or an aryl ester group having 1 or 2 rings, e.g., benzoate; a heteroaryl group having 1 or 2 rings, e.g., benzoxazole, benzthiazole, benzimidazole or benztriazole; or hydrogen or an enzyme-cleavable or chemically cleavable group Z as defined herein, with
 50 at least one of R₃ being an enzyme-cleavable group if no other substituent on the dioxetane ring is a fluorophore group having an enzyme-cleavable substituent. Furthermore, all of the R₃ groups together can form a ring which can be substituted or unsubstituted.

The symbol T represents a stabilizing group that prevents the dioxetane compound from decomposing before the bond in the labile ring substituent, e.g., the enzyme-cleavable bond in an enzyme-cleavable
 55 group, on the light-emitting fluorophore-forming fluorescent chromophore group is intentionally cleaved, such as an unsubstituted or substituted cycloalkyl, aryl, including fused aryl, or heteroaryl group, e.g., an unsubstituted cycloalkyl group having from 6 to 12 ring carbon atoms, inclusive; a substituted cycloalkyl group having from 6 to 12 ring carbon atoms, inclusive, and having one or more substituents which can be

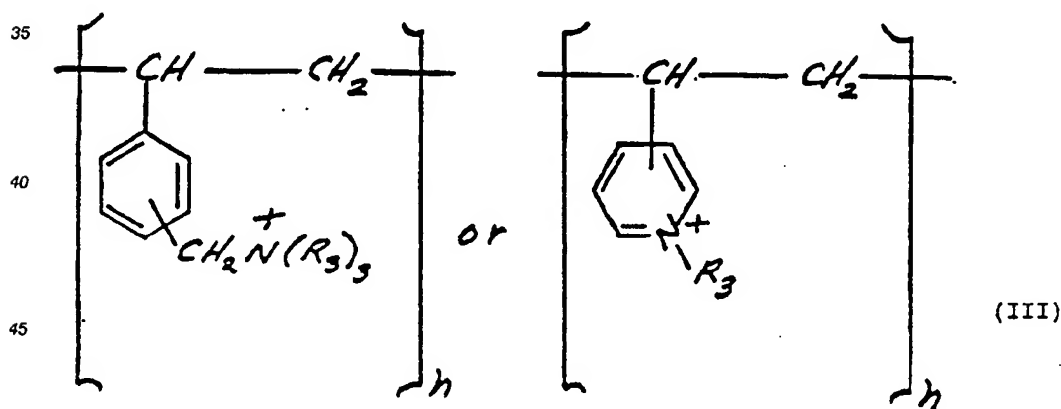
an alkyl group having from 1 to 7 carbon atoms, inclusive, or a heteroatom group which can be an alkoxy group having from 1 to 12 carbon atoms, inclusive, such as methoxy or ethoxy, a substituted or unsubstituted aryloxy group, such as phenoxy or carboxyphenoxy, or an alkoxyalkyloxy group, such as methoxyethoxy or polyethyleneoxy, or a cycloalkylidene group bonded to the dioxetane ring through a spiro linkage and having from 6 to 12 carbon atoms, inclusive, or a fused polycycloalkylidene group bonded to the dioxetane ring through a spiro linkage and having two or more fused rings, each having from 5 to 12 carbon atoms, inclusive, e.g., an adamant-2-ylidene group.

One or more of the substituents R_1 , R_2 and T can also include a substituent which enhances the water solubility of the 1,2-dioxetane, such as a carboxylic acid, e.g., acetic acid; sulfonic acid, e.g., methanesulfonic acid or ethanesulfonic acid; or quaternary amino salt group, e.g., ammonium bromide, and at least one of R_1 and R_2 , and preferably R_2 , is one of the above-described light-emitting fluorophore-forming chromophore group containing an enzyme-cleavable group, and preferably an enzyme-cleavable phosphate ester group.

Included among the labile ring substituents which can be positioned on a fluorophore group to make up the fluorophore moieties of this invention are substituents which, as indicated above, are cleaved to yield an anion, e.g., an oxygen anion, a sulfur anion, or a nitrogen anion such as a sulfonamido anion. Such substituents can be chemically cleavable: a hydroxyl group, an alkanoyl or aroyl ester group, or an alkyl or aryl silyloxy group, for example, but preferably are enzymatically cleavable. Enzymatically cleavable substituents include phosphate ester groups represented by the general formula:



wherein M^+ represents a cation such as alkali metal, e.g., sodium or potassium; ammonium, or a $\text{C}_1\text{-C}_7$ alkyl, aralkyl or aromatic quaternary ammonium cation, $\text{N}(\text{R}_7)_4^+$ in which each R_7 can be alkyl, e.g., methyl or ethyl, aralkyl, e.g., benzyl, or form part of a heterocyclic ring system, e.g., pyridinium. The disodium salt is particularly preferred. Such quaternary ammonium cations can also be connected through one of their quaternizing groups to a polymeric backbone, viz.



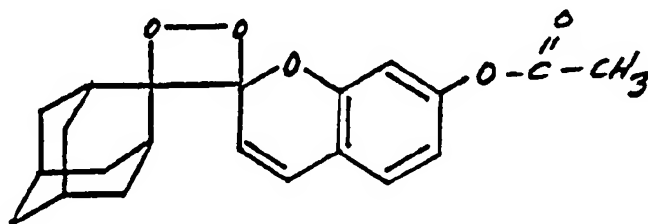
where n is greater than 1, or can be part of a polyquaternary ammonium salt.

Enzymatically cleavable substituents also include enzyme-cleavable alkanoyloxy groups, e.g., an acetate ester group, or an enzyme-cleavable oxacarboxylate group, 1-phospho-2,3-diacylglyceride group, 1-thio-D-glucoside group, adenosine triphosphate analog group, adenosine diphosphate analog group, adenosine monophosphate analog group, adenosine analog group, α -D-galactoside group, β -D-galactoside group, α -D-glucoside group, β -D-glucoside group, α -D-mannoside group, β -D-mannoside group, β -D-fructofuranoside group, β -D-glucosiduronate group, p-toluenesulfonyl-L-arginine ester group or p-toluenesulfonyl-L-arginine amide group.

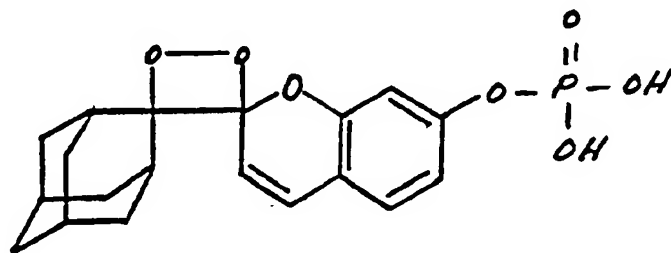
The improved methods of using chemiluminescent 1,2-dioxetanes of this invention are particularly useful when the dioxetanes are employed as the means of identifying or quantifying several analytes using otherwise art-recognized immunoassays, such as those hitherto employed to detect an enzyme or a member of a specific binding pair, e.g., an antigen-antibody pair or a nucleic acid paired with a probe capable of binding to all or a portion of the nucleic acid. Such assays include immunoassays used to detect a hormone such as β -chorionic gonadotropin (β -HCG), thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), luteinizing hormone (LH) or the like, cell surface receptor assays, and nucleic acid probe assays used to detect viruses, e.g., HIV or HTLV III, herpes simplex virus (HSV), human papilloma virus (HPV), and cytomegalovirus (CMV), or bacteria, e.g., E.Coli., and histocompatibility assays; for typical assay protocols see working examples I and II, infra. The improved methods of this invention can also be used in assays for chemical analytes, such as, potassium or sodium ions, or in assays for substances such as *inter alia*, cholesterol and glucose in which the analytes are caused to decompose, for example using an enzyme such as cholesterol oxidase or glucose oxidase, to form a substance, e.g., hydrogen peroxide, capable in combination with another reagent of causing the chemiluminescent compound to decompose.

As noted above, by using two or more chemiluminescent 1,2-dioxetanes that each emit light of a different wavelength from the others, or by using one or more of these different colored light-emitting chemiluminescent 1,2-dioxetanes with one or more other chemiluminescent compounds which emit light of yet other wavelengths, each of such compounds being structured so as to be decomposable by a different means, this invention enables multi-channel assays be designed in which different cleaving means, and especially two or more different enzymes, attached to or associated with two or more different analytes will, by cleaving different cleavable dioxetane substituents, induce the emission of light of a different wavelength for each analyte being assayed.

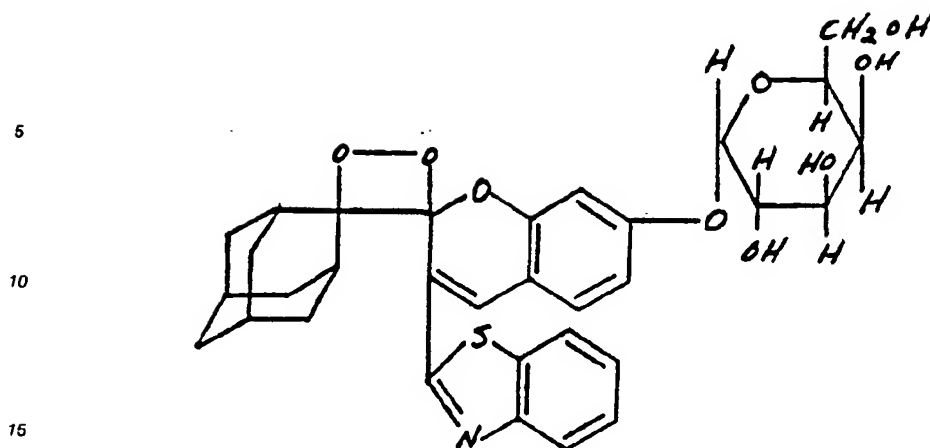
3-(2'-Spiroadamantane)-4-(7''-acetoxy)benzo-2H-pyran-2'-yl-1,2-dioxetane [dispiro(adamantane-2)-3''-(1',2'-dioxetane)-4',2''-(7''-acetoxy-3''-chromene)],



for example, when cleaved with a carboxylesterase, will emit light of 450 nm. (blue), 3-(2'-spiroadamantane)-4-(7''-phosphoryloxy-4''-trifluoromethyl)benzo-2H-pyran-2'-yl-1,2-dioxetane,



when cleaved with an alkaline phosphatase, will emit light of 480 nm. (cyan, i.e., blue green), and 3-(2'-spiroadamantane)-4-(3''-benzothiazol-2-yl-7''- β -galactosyloxy)benzo-2H-pyran-2'-yl-1,2-dioxetane,



when cleaved with β -galactosidase, will emit light of 515 nm. (green). A simultaneous assay for HLH, FSH and β -HCG, or any two of them, can hence be designed using these three chemiluminescent substances, or any two of them, to produce light emissions of a different color for each of the analytes.

Such an assay can, for example, be a simultaneous sandwich two antibody capture enzyme immunoassay in which a serum or urine sample containing a mixture of analytes: HLH, FSH and β -HCG, for example, or any two of them, is added to a coated matrix containing capture antibodies specific for HLH, FSH and β -HCG and incubated. Next, enzyme labeled antibodies: anti HLH labeled with β -galactosidase, anti FSH labeled with alkaline phosphatase and anti β -HCG labeled with acetylcholinesterase, are added, followed by, e.g., a mixture of the aforementioned three chemiluminescent 1,2-dioxetane substrates: the acetoxybenzopyranyl dioxetane cleavable with acetylcholinesterase, the phosphoryloxybenzopyranyl dioxetane cleavable with alkaline phosphatase and the β -galactosyloxybenzopyranyl dioxetane cleavable with β -galactosidase. The resulting light emissions can be detected either with three different monochromators or on black and white photographic film with three different color filters, with the intensity of the light emissions being a function of the various analyte concentrations.

A homogeneous assay using, e.g., these same three chemiluminescent 1,2-dioxetane substrates can be carried out by first adding a mixture of the analytes (Ag_1 , Ag_2 , Ag_3) to a mixture of specific Anti Ag_1 , Anti Ag_2 and Anti Ag_3 antibodies and small quantities of each of the three analytes bound to the three different enzymes: Ag_1 - β -galactosidase, Ag_2 -alkaline phosphatase, Ag_3 -acetylcholinesterase, and incubating.

Since in anti Ag_1 Ag_1 - β -galactosidase, Anti Ag_2 Ag_2 -alkaline phosphatase and Anti Ag_3 Ag_3 -acetylcholinesterase complexes the enzyme will be inactivated and hence unable to induce luminescence, only enzyme labeled antigens that are unbound will cleave the substrates to emit light. The emitted light can be detected in the same manner as in the above-described sandwich assay. Since there is a competition between native antigens and enzyme labeled antigens, the intensity of the light emitted will be a function of unbound labeled antigens, and thus will correspond to the concentrations of the analytes measured.

Light of various colors emitted when using the improved methods of this invention to identify or quantify various analytes can also be used to make a permanent record of such emissions on color photographic emulsions as well as on specially sensitized black and white high speed films. And, these improved methods can be used to achieve a matched response by detectors: charged coupled devices (CCD's) or silicon photodiodes, for example, having maximum sensitivity for a color other than blue, e.g., green or red. Further, by using chemiluminescent 1,2-dioxetanes together with a light absorbing/light shifting auxiliary fluorophore/light enhancer substance which absorbs light of one wavelength and in turn emits light of a different wavelength, e.g., a phycobiliprotein (phycobiliproteins are naturally-occurring substances in which a fluorophore is bonded to a protein), such as phycocyanine or phycoerythrin, that will absorb the green light emitted by one such substance that emits light in this region of the spectrum and reemit this light as red light, matched responses by color photographic emulsions that exhibit a poor response to blue light, a better response to green light but the best response to red light can also be achieved.

Besides the phycobiliproteins, other auxiliary fluorophores extraneous to the light-emitting fluorophores produced by the decomposition of the chemiluminescent 1,2-dioxetane compounds used in the method of this invention that will accept energy, especially light, from these light-emitting fluorophores and in turn emit detectable energy, again preferably light, can be used when practicing this invention. Among such auxiliary fluorophores that can be used, alone or in combination, are the following substances whose residues can be

present in known chemiluminescent 1,2-dioxetanes, as fluorescent chromophore groups:

- anthracene and anthracene derivatives, e.g., 9,10-diphenylanthracene, 9-methylantracene, 9-anthracene carboxaldehyde, anthrylalcohols and 9-phenylantracene;
- rhodamine and rhodamine derivatives, e.g., rhodols, tetramethyl rhodamine, tetraethyl rhodamine, diphenyldimethyl rhodamine, diphenyldiethyl rhodamine and dinaphthyl rhodamine;
- fluorescein and fluorescein derivatives, e.g., 5-iodoacetamido fluorescein, 6-iodoacetamido fluorescein and fluorescein-5-maleimide;
- coumarin and coumarin derivatives, e.g., 7-dialkylamino-4-methylcoumarin, 4-bromomethyl-7-methoxycoumarin and 4-bromomethyl-7-hydroxy coumarin;
- erythrosin and erythrosin derivatives, e.g., hydroxy erythrosins, erythrosin-5-iodoacetamide and erythrosin-5-maleimide;
- acridine and acridine derivatives, e.g., hydroxy acridines and 9-methyl acridine;
- pyrene and pyrene derivatives, e.g., N-(1-pyrene) iodoacetamide, hydroxy pyrenes and 1-pyrenemethyl iodoacetate;
- stilbene and stilbene derivatives, e.g., 6,6'-dibromostilbene and hydroxy stilbenes;
- naphthalene and naphthalene derivatives, e.g., 5-dimethylamino naphthalene-1-sulfonic acid and hydroxy naphthalenes;
- nitrobenzoxadiazoles and nitrobenzoxadiazole derivatives, e.g., hydroxy nitrobenzoxadiazoles, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole, 2-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) methylaminoacetaldehyde and 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminohexanoic acid;
- quinoline and quinoline derivatives, e.g., 6-hydroxyquinoline and 6-aminoquinoline;
- acridine and acridine derivatives, e.g., N-methylacridine and N-phenylacridine;
- acidoacridine and acidoacridine derivatives, e.g., 9-methylacidoacridine and hydroxy-9-methylacidoacridine;
- carbazole and carbazole derivatives, e.g., N-methylcarbazole and hydroxy-N-methylcarbazole;
- fluorescent cyanines, e.g., DCM (a laser dye), hydroxy cyanines, 1,6-diphenyl-1,3,5-hexatriene, 1-(4-dimethyl aminophenyl)-6-phenylhexatriene and the corresponding 1,3-butadienes;
- carbocyanines and carbocyanine derivatives, e.g., phenylcarbocyanine and hydroxy carbocyanines;
- pyridinium salts, e.g., 4-(4-dialkyldiaminostyryl)N-methyl pyridinium iodate and hydroxy-substituted pyridinium salts;
- oxonols; and
- resorofins and hydroxy resorofins.

When such auxiliary fluorophores are bonded to a chemiluminescent compound, they are preferably bonded to the portion of the chemiluminescent compound that, upon decomposition, forms a fragment containing the fluorophore portion of the chemiluminescent compound's molecule. In this way energy transfer is enhanced due to the two fluorophores being in close proximity to one another and by beneficial spatial arrangements provided by the rigidity of the microenvironment. Auxiliary fluorophores that are insoluble or partially insoluble in aqueous medium can be solubilized by first grafting them onto solubilizing molecules, e.g., water soluble oligomer or polymer molecules.

And, in all cases, enhancement of the intensity of the light emitted by decomposition of the chemiluminescent 1,2-dioxetane compounds used in the improved methods of this invention carried out in aqueous media can be achieved.

In order that those skilled in the art can more fully understand this invention, the following examples are set forth. These examples are given solely for purposes of illustration, and should not be considered as expressing limitations unless so set forth in the appended claims. All parts and percentages are by weight, unless otherwise stated.

EXAMPLE I

A dual channel assay for Human Chorionic Gonadotropins (β -chain), β -HCG, and Human Luteinizing Hormones, HLH, is carried out as follows:

MATERIALS:

A round nylon membrane (approximately 1 inch in diameter) containing two sets of covalently immobilized capture monoclonal antibodies is used, one set for β -HCG available from Medix Biotech, Anti HCG, Cat. No. H298-01, and the second for HLH also available from Medix Biotech, Anti LH, cat. No. L461-09. This nylon membrane is stored in a foil pouch until used.

Mouse monoclonal anti β -HCG available from Medix Biotech, Cat. No. H298-12, is conjugated with alkaline phosphatase using the glutaraldehyde coupling procedure [Voller, A., et. al., *Bull. World Health Org.*, 53, 55 (1976)] and used as a detection antibody for β -HCG.

Mouse monoclonal anti HLH available from Medix Biotech, Cat. No. L461-03, is conjugated to carboxylesterase also using the glutaraldehyde coupling procedure referenced above, and used as a detection antibody for HLH.

The substrate buffer solution contains 0.05M carbonate, 1mM $MgCl_2$, 0.1% by weight BSA (pH = 0.5) and 3-(2'-spiroadamantane)-4-methoxy-4(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt, (50 μ g/ml), and 3-(2'-spiroadamantane)-4-(3''-benzothiazol-2-yl-7''- β -galactosyloxy)benzo-2H-pyran-2'-yl-1,2-dioxetane, (50 μ g/ml) as the chemiluminescent substrates.

The wash buffer contains 0.05M carbonate, 1mM $MgCl_2$ and 2% by weight BSA (pH = 9.5).

ASSAY PROCEDURE:

Five drops of a previously collected urine sample are placed onto the center of the assay membrane and allowed to soak into the membrane. Next, five drops of a solution containing β -HCG and HLH conjugated detection antibodies at a concentration of 0.01 millimolar are added to the assay membrane. The liquid is allowed to soak in for at least one minute. Six drops of the wash buffer are slowly added and allowed to soak in and drain for 30 to 60 seconds. Then, five drops of the buffer solution containing chemiluminescent substrates are added and allowed to soak in for approximately one minute.

The assay membrane is placed in a camera luminometer device equipped with pre-exposed calibration scales for β -HCG and LH.

The chemiluminescent light emission generated as a function of the enzymes, alkaline phosphatase and carboxyl esterase, is imaged through a mask containing two narrow band pass filters (approximately 1cm in diameter). Kodak Wratten Gelatin Filter No. 115 is used to image green emission from the benzopyranyl 1,2-dioxetane substrate, and Kodak Wratten Filter No. 47B is used to isolate the blue emission from the phenyl dioxetane.

The relative levels of β -NCG and HLH present in the sample are determined by a comparison of the appropriate imaged spot brightness with relevant calibration scales.

EXAMPLE II

A three-channel analysis for Herpes Simplex Virus, (HSV), Cytomegalovirus, (CMV), and Human Papilloma Virus, (HPV) is carried out as follows:

MATERIALS:

"Gene Screen Plus", a positively charged nylon membrane (Dupont NEN Products) is used for hybridization.

The following buffers are used for the assay:

HSV DNA PROBE ASSAY

Materials and Buffers:

Membrane: Gene Screen Plus membrane.
 Buffers: Denaturation Buffer, 0.5 M NaOH
 Neutralization Buffer, 0.4M NaH_2PO_4 (pH = 2.0)
 Loading Buffer, 1 part Denaturation Buffer
 1 part Neutralization Buffer
 Membrane Wetting Buffer 0.4M Tris (pH = 7.5)

Membrane Prehybridization Buffer:

	<u>Substance</u>	<u>Final Concentration</u>
5	0.5 ml 100 X Denhardt's solution	5 X
	0.5 ml 10% SDS	0.5%
	2.5 ML 20 x SSPE	5 X
10	2.0 mg denatured, sonicated salmon sperm DNA	200 µg/ml
	<u>ddH₂O</u>	
15	10ml	

Membrane Hybridization Buffer:

	<u>Substance</u>	<u>Final Concentration</u>
20	0.5 ml 100 X Denhardt's solution	5 X
	0.5 ml 10% SDS	0.5%
	2.5 ml 20 X SSPE	5 X
25	2.0 mg salmon sperm DNA	200 µg/ml
	2.0 ml 50% Dextran sulfate	10%
	-- ddH ₂ O	
	10 ml	

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Wash Buffer I:

35	1 X SSPE/0.1% SDS
	20ml 20 X SSPE
	4ml 10% SDS
	<u>376ml</u> ddH ₂ O
	400ml

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Wash Buffer II:

45	0.1 X SSPE/0.1% SDS preheated to wash temperature indicated on Technical Data Sheet.
	2ml 20 X SSPE
	4ml 20% SDS
	<u>394ml</u> ddh ₂ O
50	400ml (heated)

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Wash Buffer III:

0.1 X SSPE/0.1% SDS
 2 ml 20 X SSPE
 4 ml 10% SDS
 394 ml ddH₂O
 400 ml

Wash Buffer IV:

3 mM Tris-HCl (pH 9.5)
 0.6 ml 1 M Trizma Base
 199.4 ml ddH₂O
 200.0 ml

Wash Buffer V:

0.1 M Trizma HCl pH 6.0
 100 X Denhart's solution

Preparation of 100 X Denhart's solution (for 100 mls):

Polyvinylpyrrolidone (2g; mol. wt. 40K; PVP-40) and 2g ficoll are dissolved at a temperature greater than 65°C but less than boiling. The solution is cooled to approximately 40°C, 2g BSA are added and the solution is brought up to the final volume of 100 ml with ddH₂O, aliquoted and stored at -20°C. BSA is easily denatured and in combination with PVP and ficoll it will not go into solution at all if it is not cooled down to -40°C. Hence, the solution is not heated over 40°C when thawing for use.

Preparation of 20 X SSC solution:

20 X SSC (for 100 ml)
 3.0 M Sodium Chloride 17.4g
 0.3 M Sodium Citrate 8.8g

The volume is brought to 100 ml and the solution filtered through a 0.45 µm nitrocellulose filter and stored at room temperature.

Preparation of 20 X SSPE solution:

20 X SSPE pH 7.4 (for 1 liter)

3.6 M NaCl

200 mM Sodium phosphate 23 g dibasic, 5.92g monobasic

20 mM EDTA 7.44g

These materials are dissolved, adjusted to pH 7.4 with 5N NaOH, brought to a volume of 1 liter and the solution is then filtered through a 0.45 µm nitrocellulose filter.

1 X TE:	
1 X TE buffer	10 mM Tris (pH 7.) 1 mM EDTA Autoclave

The substrate buffer solution contains 0.05M carbonate, 1 mM MgCl₂, 0.1% by weight BSA (Ph = 9.5) and 3-(2'-spiroadamantane)-4-methoxy-4-(3'-acetoxy)phenyl-1,2-dioxetane disodium salt (50 mg/ml), 3-(2'-spiroadamantane)-4-(7''-phosphoryloxy-4''-trifluoromethyl)benzo-2H-pyran-2'-yl-1,2-dioxetane, (50 mg/ml) and 3-(2'-spiroadamantane)-4-(3''-benzothiazol-2-yl-7''-β-galactosyloxy)benzo-2H-pyran-2'-yl-1,2-dioxetane (50 mg/ml) as the chemiluminescent substrates.

ASSAY PROCEDURE:

Samples (50 μl) containing DNA are denatured by incubation for 10 minutes at room temperature in 200 μl of Denaturation Buffer. Ice cold Neutralization Buffer (250 μl) is then added, and the samples placed on ice.

Nylon membrane is soaked for 15 minutes in Wetting Buffer and then inserted into a vacuum manifold device producing 2 cm diameter spots. Loading Buffer, (200 μl) is then aspirated through each well. The denatured DNA samples are then added to each well and aspirated through the membrane. The manifold is then disassembled and the DNA fixed to the membrane using a UV Transilluminator for 5 minutes. The membrane is then incubated in Prehybridization Buffer at 70 °C for 1 hour.

Dots of membrane from the region to which the sample DNA is applied are punched out and inserted into tubes for the remaining steps of the assay. The following enzyme labeled probes are used: probe for HSV labeled with alkaline phosphatase; probe for HPV labeled with β-galactosidase; probe for CMV labeled with carboxylesterase.

The enzyme labeled probes (50 ng of each probe in 200 μl of Hybridization Buffer per tube) are added to each tube and incubated for 2 hours at 70 °C. The Hybridization Buffer is removed and 400 μl of Wash Buffer I added and the tubes agitated for 10 minutes at room temperature. Washing is continued by first washing with 400 μl of Wash Buffer II at 50 °C for 30 minutes; then with 400 μl of Wash Buffer III at room temperature for 10 minutes; and then with 200 μl of Wash Buffer IV at room temperature.

The membrane is subsequently rinsed with Wash Buffer V at pH 6.0 and placed on a piece of transparent Mylar polyester film. Then, 200 μl of the Substrate Buffer is added and allowed to soak in.

The assay membrane is placed in a camera luminometer device equipped with pre-exposed calibration scales for HSV, HPV and CMV.

The chemiluminescent light emission generated as a function of the enzymes -- alkaline phosphatase, carboxyl esterase and β-galactosidase -- is imaged through a mask containing three narrow bandpass Kodak Wrattan gelatin filters (approximately 1cm in diameter), which isolate the blue emission from the phenyl phosphate dioxetane, the cyan emission from the phosphoryloxytrifluoromethylbenzopyranyl dioxetane and the green emission from the galactosyloxybenzopyranyl dioxetane, respectively.

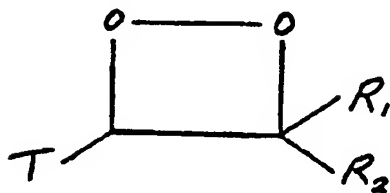
The relative levels of HSV, HPV and CMV present in the sample are determined by a comparison of the appropriate image brightness with the relevant calibration scale.

The above discussion of this invention is directed primarily to preferred embodiments and practices thereof. It will be readily apparent to those skilled in the art that further changes and modifications in the actual implementation of the concepts described herein can easily be made without departing from the spirit and scope of the invention as defined by the following claims.

Claims

1. A process in which light of different wavelengths is simultaneously released from two or more enzymatically decomposable chemiluminescent 1,2-dioxetane compounds, said compounds being configured, by means of the inclusion of a different light emitting fluorophore in each of them, to each emit light of said different wavelengths, which comprises decomposing each of said compounds by means of a different enzyme.

2. The process of claim 1 in which each of said compounds is represented by the general formula:



wherein R_1 represents hydrogen, or a bond when R_2 represents a substituent bonded to the dioxetane ring through a spiro linkage, or an organic substituent that does not interfere with the production of light and that satisfies the valence of the dioxetane ring carbon atom to which it is attached, or a light-emitting fluorophore-forming fluorescent chromophore group bonded to the dioxetane ring through a single bond or a spiro linkage, to which an enzyme-cleavable group is bonded, R_2 represents hydrogen, or a bond when R_1 represents a substituent bonded to the dioxetane ring through a spiro linkage, or a light-emitting fluorophore-forming fluorescent chromophore group bonded to the dioxetane ring through a single bond or a spiro linkage, to which an enzyme-cleavable group is bonded, at least one of R_1 and R_2 being such light-emitting fluorophore-forming fluorescent chromophore group, and T represents a stabilizing group that prevents the dioxetane compound from decomposing before the bond in the enzyme-cleavable group is intentionally cleaved.

3. The process of claim 2 in which the process carried out is a step in an immunoassay.
4. The process of claim 3 in which the immunoassay is for the detection of specific binding pairs comprising an antigen and an antibody.
5. The process of claim 3 in which the labels used in the assay are enzymes.
6. The process of claim 3 in which the labels used in the assay are the chemiluminescent 1,2-dioxetane compounds.
7. The process of claim 3 in which the immunoassay is for the detection of an enzyme.
8. The process of claim 3 in which the immunoassay is for the detection Of hormones.
9. The process of claim 2 in which the process carried out is a step in a chemical assay.
10. The process of claim 9 in which the chemical assay is for the detection of chemical substances which, during the assay, are caused to decompose to form substances capable of causing the chemiluminescent 1,2-dioxetane compounds to decompose.
11. The process of claim 10 in which one of the chemical substances is glucose.
12. The process of claim 10 in which one of the chemical substances is cholesterol.
13. The process of claim 2 in which the process carried out is a nucleic acid probe assay.
14. The process of claim 13 in which the nucleic acid probe assay is for the detection of viruses.
15. The process of claim 2 in which the process carried out is a histocompatibility assay.
16. The process of claim 2 in which the process carried out is a technique for studying the microstructure of a macromolecule.
17. The process of claim 2 in which the process carried out is a multi-channel assay carried out in the presence of at least two of the chemiluminescent 1,2-dioxetane compounds as recited in claim 2 as

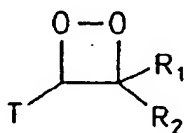
substrates, each of which upon decomposition emits light of a different wavelength from the other(s) and each of which has a labile ring substituent cleavable by a different means from the other(s).

18. The process of claim 2 in which the process carried out is a multi-channel assay carried out in the presence of at least one of the chemiluminescent 1,2-dioxetane compounds as recited in claim and at least one other chemically decomposable chemiluminescent compound as substrates, each of which chemiluminescent 1,2-dioxetane compounds and other chemiluminescent compounds emit light of a different wavelength from the other(s).

10 Patentansprüche

1. Verfahren, bei dem Licht unterschiedlicher Wellenlängen von zwei oder mehreren enzymatisch zersetz-
baren, chemolumineszierenden 1,2-Dioxetan-Verbindungen freigesetzt wird, wobei die Verbindungen
durch Einschluß von, unterschiedliches Licht ermittlendem Fluorophor in jede von ihnen, so konfigu-
riert sind, daß jeweils Licht der unterschiedlichen Wellenlängen emittiert wird, wobei das Verfahren die
Zersetzung jeder der Verbindungen mittels eines unterschiedlichen Enzyms umfaßt.

2. Verfahren nach Anspruch 1, wobei jede der Verbindungen durch die allgemeine Formel



dargestellt wird,

wobei R_1 Wasserstoff, oder, wenn R_2 einen Substituenten darstellt, der an den Dioxetan-Ring durch eine Spiro-Verknüpfung gebunden ist, eine Bindung oder einen organischen Substituenten, der die Lichterzeugung nicht störend beeinflußt und der die Valenz des Dioxetanring-Kohlenstoffatoms, an das er gebunden ist, absättigt, oder eine lichtemittierende, fluorophorbildende, fluoreszierende, chromophore Gruppe, die an den Dioxetanring durch eine Einfachbindung oder eine Spiro-Verknüpfung gebunden ist, und an welche eine enzymatisch abspaltbare Gruppe gebunden ist, darstellt,

R_2 Wasserstoff, oder, wenn R_1 einen Substituenten darstellt, der an den Dioxetan-Ring durch eine Spiro-Verknüpfung gebunden ist, eine Bindung oder eine lichtemittierende, fluorophorbildende, fluoreszierende, chromophore Gruppe, die an den Dioxetanring durch eine Einfachbindung oder eine Spiro-Verknüpfung gebunden ist und an welche eine enzymatisch abspaltbare Gruppe gebunden ist, darstellt, wobei mindestens einer der beiden Reste R_1 und R_2 eine solche lichtemittierende, fluorophorbildende, fluoreszierende, chromophore Gruppe ist, und T eine stabilisierende Gruppe darstellt, die die Dioxetan-Verbindung vor Zersetzung schützt, bevor die enzymatisch abspaltbare Gruppe beabsichtigtweise abgespalten wird.

3. Verfahren nach Anspruch 2, wobei das durchgeführte Verfahren ein Schritt in einem Immunoassay ist.

4. Verfahren nach Anspruch 3, wobei der Immunoassay zum Nachweis eines spezifischen Bindungs-Paares, welches ein Antigen und einen Antikörper umfaßt, dient.

5. Verfahren nach Anspruch 3, wobei die im Assay verwendeten Marker Enzyme sind.

6. Verfahren nach Anspruch 3, wobei die im Assay verwendeten Marker die chemolumineszierenden 1,2-Dioxetane sind.

7. Verfahren nach Anspruch 3, wobei der Immunoassay zum Nachweis eines Enzyms dient.

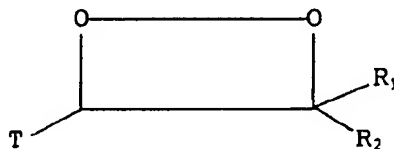
8. Verfahren nach Anspruch 3, wobei der Immunoassay zum Nachweis von Hormonen dient.

9. Verfahren nach Anspruch 2, wobei das durchgeführte Verfahren ein Schritt in einem chemischen Assay ist.

10. Verfahren nach Anspruch 9, wobei der chemische Assay zum Nachweis von chemischen Substanzen dient, die während des Assays dazu veranlaßt werden, sich zu zersetzen, um dabei Substanzen zu bilden, die in der Lage sind, die Zersetzung der chemolumineszierenden 1,2-Dioxetane herbeizuführen.
11. Verfahren nach Anspruch 10, wobei eine der chemischen Substanzen Glukose ist.
12. Verfahren nach Anspruch 10, wobei eine der chemischen Substanzen Cholesterol ist.
13. Verfahren nach Anspruch 2, wobei das durchgeführte Verfahren ein Nucleinsäure-Sondenassay ist.
14. Verfahren nach Anspruch 13, wobei das Nucleinsäure-Sondenassay für den Nachweis von Viren dient.
15. Verfahren nach Anspruch 2, wobei das durchgeführte Verfahren ein Histokompatibilitäts-Assay ist.
16. Verfahren nach Anspruch 2, wobei das durchgeführte Verfahren eine Technik zum Studieren der Mikrostruktur eines Makromoleküls ist.
17. Verfahren nach Anspruch 2, wobei das durchgeführte Verfahren ein Multikanal-Assay ist, das in Gegenwart von mindestens zwei der chemolumineszierenden 1,2-Dioxetan-Verbindungen, wie in Anspruch 2 aufgeführt, als Substraten durchgeführt wird, wobei jede dieser Verbindungen bei der Zersetzung Licht einer Wellenlänge emittiert, die sich von der (den) anderen unterscheidet, und wobei jede einen labilen Ring-Substituenten besitzt, der durch Mittel abspaltbar ist, die von der (den) anderen verschieden sind.
18. Verfahren nach Anspruch 2, wobei das durchgeführte Verfahren ein Multikanal-Assay ist, das in Gegenwart von mindestens einer der chemolumineszierenden 1,2-Dioxetan-Verbindungen, wie in Anspruch 2 aufgeführt und mindestens einer anderen chemisch zersetzbaren, chemolumineszierenden Verbindung als Substrate durchgeführt wird, wobei jede der chemolumineszierenden 1,2-Dioxetan-Verbindungen und der anderen chemolumineszierenden Verbindungen Licht einer Wellenlänge emittiert, die sich von der (den) anderen unterscheidet.

Revendications

1. Procédé dans lequel de la lumière de différentes longueurs d'onde est libérée simultanément par deux ou plus de deux composés de 1,2-dioxétane chimioluminescents décomposables par une enzyme, lesdits composés étant configurés, grâce à l'inclusion d'un fluorogène luminescent différent dans chacun d'eux, de sorte que chacun émette une lumière desdites longueurs d'onde différentes, qui comprend la décomposition de chacun desdits composés à l'aide d'une enzyme différente.
2. Procédé de la revendication 1 dans lequel chacun desdits composés est représenté par la formule générale :



- où R_1 représente de l'hydrogène, ou une liaison lorsque R_2 représente un substituant lié au cycle dioxétane par une liaison en spirale, ou un substituant organique qui ne perturbe pas la production de lumière et qui respecte la valence de l'atome de carbone du cycle dioxétane auquel il est fixé, ou un groupe chromophore fluorescent et luminescent formant un fluorogène, lié au cycle dioxétane par une liaison simple ou une liaison en spirale, auquel un groupe pouvant être coupé par une enzyme est lié ; R_2 représente de l'hydrogène, ou une liaison lorsque R_1 représente un substituant lié au cycle dioxétane par une liaison en spirale, ou un groupe chromophore fluorescent et luminescent formant un fluorogène lié au cycle dioxétane par une liaison simple ou une liaison en spirale, auquel un groupe pouvant être coupé par une enzyme est lié ; au moins l'un parmi R_1 et R_2 étant ce groupe

chromophore fluorescent et luminescent formant un fluorogène ; et T représente un groupe stabilisant qui empêche le composé de dioxétane de se décomposer avant la coupure intentionnelle de la liaison du groupe pouvant être coupé par une enzyme.

- 5 3. Procédé de la revendication 2 dans lequel le procédé suivi est une étape d'un essai immunologique.
4. Procédé de la revendication 3 dans lequel l'essai immunologique sert à détecter des doublets de liaison spécifiques comprenant un antigène et un anticorps.
- 10 5. Procédé de la revendication 3 dans lequel les marqueurs utilisés dans l'essai sont des enzymes.
6. Procédé de la revendication 3 dans lequel les marqueurs utilisés dans l'essai sont les composés de 1,2-dioxétane chimioluminescents.
- 15 7. Procédé de la revendication 3 dans lequel l'essai immunologique sert à détecter une enzyme.
8. Procédé de la revendication 3 dans lequel l'essai immunologique sert à détecter des hormones.
9. Procédé de la revendication 2 dans lequel le procédé suivi est une étape d'un essai chimique.
- 20 10. Procédé de la revendication 9 dans lequel l'essai chimique sert à détecter des substances chimiques que l'on fait décomposer, au cours de l'essai, pour former des substances capables de provoquer la décomposition des composés de 1,2-dioxétane chimioluminescents.
- 25 11. Procédé de la revendication 10 dans lequel l'une des substances chimiques est le glucose.
12. Procédé de la revendication 10 dans lequel l'une des substances chimiques est le cholestérol.
- 30 13. Procédé de la revendication 2 dans lequel le procédé suivi est un essai d'exploration des acides nucléiques.
14. Procédé de la revendication 13 dans lequel l'essai d'exploration des acides nucléiques sert à détecter les virus.
- 35 15. Procédé de la revendication 2 dans lequel le procédé suivi est un essai d'histocompatibilité.
16. Procédé de la revendication 2 dans lequel le procédé suivi est une technique d'étude de la microstructure d'une macromolécule.
- 40 17. Procédé de la revendication 2 dans lequel le procédé suivi est un essai à voies multiples se déroulant en présence d'au moins deux des composés de 1,2-dioxétane chimioluminescents tels qu'énoncés dans la revendication 2 comme substrats, dont chacun émet une lumière d'une longueur d'onde différente de l'autre (des autres) quand il se décompose, et contient un substituant labile du cycle pouvant être coupé par un moyen différent de l'autre (des autres).
- 45 18. Procédé de la revendication 2 dans lequel le procédé suivi est un essai à voies multiples se déroulant en présence d'au moins l'un des composés de 1,2-dioxétane chimioluminescents tels qu'énoncés dans la revendication 2 et d'au moins un autre composé chimioluminescent chimiquement décomposable comme substrats ; les composés de 1,2-dioxétane chimioluminescents et les autres composés
- 50 chimioluminescents émettant chacun une lumière de longueur d'onde différente par rapport à l'autre (aux autres).